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(54) Title: THE USE OF POLYMER ADSORBENT PARTICLES IN DNA SEPARATION

(57) Abstract: A method for the separation of a nucleic acid in a liquid sample, comprises passing the sample through a bed of adsorbent particles of a fluoropolymer, and elution of a desired fraction, wherein the method comprises the use of an ion-pairing component.

THE USE OF POLYMER ADSORBENT PARTICLES IN DNA SEPARATION

Field of invention

The invention relates to the use of polymer adsorbent particles as a stationary phase for carrying out selective DNA separations.

Background of the Invention

Support materials for use in high productivity liquid chromatography must be mechanically strong in order to withstand operation at high rates of flow under high pressures. Moreover, they must be stable over the wide range of pH to which such materials are subjected during normal operation and regeneration. The stability of the polymeric particles in its environment allows it. to withstand degradation and decomposition. Properties of particular importance for chromatographic media are: (1) surface chemistry of the particles; (2) surface area; (3) pore volume and availability; (4) pore diameter; and (5) particle diameter.

WO-A-00/77081 and US-A-604246 (the contents of which are incorporated herein by reference) disclose the manufacture of improved fluorinated particles having adsorbent properties for superior performance as the stationary phase for use in chromatographic separations. The process comprises the steps of:

(1) forming a water-insoluble solution of organic compounds comprising a monomer selected from C_{2-4} alkylene glycol esters of a C_{3-6} acrylic acid or divinyl. benzene; a polyfluorinated vinyl monomer; a free radical initiator; and a water-insoluble, organic solvent-soluble porogenic material, the weight ratio of the comonomers to porogenic material being from 0.5:1 to 2:1;

(2) forming a dilute solution of a dispersing agent in water from which any oxygen has been purged with inert gas;

(3) with agitation and inert gas purging, rapidly dispersing the water-insoluble solution of organic compounds from step (1) into the dilute aqueous solution from step (2) and, as necessary, adjusting the temperature of the dispersion to 30-90^{0}C to initiate copolymerisation of the monomers, the level of mixing energy being sufficient to disperse the water-insoluble solution of organic compounds in the solution from step (2) in the form of liquid droplets having an average diameter of no more than 10-300 micrometers, at least 90% of the droplets being within 40% above or below the average mean particle diameter;

(4) continuing the agitation and oxygen purging of the dispersion from step (3) for a time sufficient to effect complete copolymerisation of the monomers and

particulation of the droplets in the form of finely divided polymer particles by precipitation of the copolymer therein;

(5) separating the finely divided copolymer particles from the polymerization reaction medium;

(6) extracting the porogenic material from the separated copolymer particles of step (5) by washing the particles with inert organic solvent, thereby forming pores within the copolymer; and

(7) drying the porous copolymer particles.

Adsorbent particles made by the process described above are disclosed as being particularly suited to use where a sample to be chromatographed comprises a mixture of macromolecules containing nucleotides, nucleosides or polypeptides, such as DNA, RNA or endotoxins.

US-A-5438129 and US-A-5625054 disclose fluorinated silica for DNA separation.

Reverse phase chromatography involves the use of a relatively non-polar stationary phase in conjunction with a very polar mobile phase that is usually water. This technique is used to separate solutes of lower polarity. Reverse phase chromatography is usually performed using silica that is coated with an organic silane to provide hydrophobicity. However, the hydrophobised silica has a severe limitation in that it cannot be used at a pH greater than 11 and cannot be cleaned with concentrated caustic soda solutions without dissolving the particles.

Summary of the Invention

WO-A-00/77081 was published after the priority date claimed for this Application. Its Example 31 discloses for the first time that a suitable polymeric medium is useful for the selective separation of DNA, using an ion-pairing component (IPC). That discovery is the basis of the present invention.

More particularly, after a plasmid is lysed with caustic, the resultant mixture may contain DNA, RNA, endotoxin and proteins. If the mixture is allowed to flow through a bed of a fluorinated polymer powder, the components are adsorbed onto the polymer. If the polymer is then treated with an aqueous solution of an ion-pairing agent at the appropriate pH, typically using an appropriate buffer, pure DNA or RNA can be preferentially eluted.

Description of the Invention

A characteristic feature of the present invention is the use of an ion-pairing component. This component has the function of neutralising the charge of the moiety

to be separated as well as the particulate medium, thereby allowing the interaction of the two to function in a type of reverse phase separation. Suitable ion-pairing components are well known to those skilled in the art. Ion-pairing agents that can be used are triethylamine salts such as the acetate, tetrabutylammonium ion, e.g. as the phosphate or acetate, dodecylsulfate ion and tetraoctylammonium ion.

In a preferred embodiment of the present invention, polar components are absent, or at least substantially absent. Thus, for example, the materials used in the invention preferably contain minimal or no polar groups such as hydroxyl, carbonyl or acidic functionality.

The fluoropolymer that is used in the invention may be of the spherical type, for example as disclosed in WO 00/77081. However, any form of fluoropolymer that has adsorbent properties can be used, whether in spherical, granular or other form. If desired, a mixture of spherical and granular fluoropolymer particles may be used, e.g. a mixture comprising 10-90% by weight of each type. It appears that such a mixture may have the effect of optimising the yield of the DNA and chromatographic flow time.

Typical fluorine-containing comonomers contain a plurality of fluorine (F) substituents. It is preferred that the fluorinated comonomer contains at least three F substituents. In addition to these restrictions on its degree of fluorination, it is desirable that the fluorinated comonomer be essentially completely insoluble in water under the polymerization temperatures encountered and essentially completely soluble in the other components of the dispersed polymerization system.

Suitable polyfluorinated comonomers are those containing active unsaturated groups such as acrylates, methacrylates, vinyl compounds, maleates and itaconates. Among the many compounds within those categories are pentafluorostyrene, bis-hexafluoroisopropyl itaconate, bis-hexafluoroisopropyl maleate, heptadecafluorodecyl acrylate, perfluorooctyl methacrylate, 2,2,3,3-tetrafluoropropyl methacrylate, mono-trifluoroethyl itaconate, 2,2,2-trifluoroethyl maleate, perfluoroctanoate, vinyl trifluoroacetate and tetrafluoroethylene.

It is preferred that any other comonomer component of the polyfluorinated copolymer for use in the invention is a fluorinated or non-fluorinated C_{2-4} alkylene glycol ester of a C_{3-6} acrylic acid (the cross-linking comonomer) or divinylbenzene. A cross-linking comonomer should have at least two unsaturated groups. Suitable comonomers are ethylene glycol dimethacrylate, 1,3-propylene glycol dimethacrylate, 1,4-butanediol dimethacrylate, ethylene glycol itaconate, ethylene glycol diacrylate, and ethylene glycol dimaleate. Divinylbenzene can also be used for this purpose.

It is important to note that the inclusion of a divinyl co-monomer will add to the physical strength and the mechanical stability of the final product. However, it is not necessary to have this crosslinking comonomer present for the fluorine-containing particle to function as a separating media for DNA plasmids. Thus, a homopolymer of polytetrafluoroethylene can provide separation.

A mixture of non-fluorinated comonomers can also be used, where one non-fluorinated comonomer has at least two vinyl groups, i.e. the cross-linking comonomer, and a third monomer, i.e. comonomer (c), is acrylic acid, methacrylic acid, or an ester of acrylic or methacrylic acid. Typical esters are the methyl, ethyl, and hydroxyethyl esters of these acids, epoxide-containing esters of these acids and amine esters of these acids. Thus, a fourth comonomer selected from comonomers (c) may be used in the synthesis. The addition of monomer (c) has little effect on the properties of the improved particles of the invention, such as stability of the particle or pore size, but may have an effect on the DNA adsorptive properties.

For example, from 1 to 30% of the cross-linker ethylene glycol dimethacrylate can be replaced with a third or fourth monomer selected from comonomers (c). These comonomers can be chosen depending on the functionality desired. For example, functional esters of acrylic and methacrylic acid can be added such as those containing hydroxyl, epoxide, amine, quaternary ammonium, sulphonic acid etc. can be used.

The polymerisation may be conducted in the presence of a source of free radicals. Preferred free radical agents include azobisisobutyronitrile (AIBN) and benzoyl peroxide (BPO).

The polyfluorinated particles of the invention can be used for chromatographic separations either with or without a coating of a hydrophilic polymer. Suitable hydrophilic polymers for use in coating the polyfluorinated particles of the invention, so that they can be functionalised, are those which are uncharged, water-soluble, non-cyclic and have a multiplicity of hydroxyl groups. Though many several such hydrophilic polymers are useful for this particular function, polyvinyl alcohol (PVA) is preferred.

The presence of a functional comonomer with pendent groups (c) facilitates the attachment of ligands for use in chromatographic separations, by obviating the use of PVA coating as a linker between the ligand and the fluorinated particulate surface, as described in US-A-5773587 and US-A-6046246, between the perfluorinated particle and the ligand. Suitable ligands can be attached to the functional fluoropolymer particles, by known means. For example, functionalised or coated polyfluorinated particles can be further functionalised by reacting suitable molecules, e.g. with the

hydroxyl groups of the PVA. Thus, strong cationic ion-exchange functionality can be provided to the particle surfaces by placing sulfonic acid groups on the surface. Likewise, strong anionic ion-exchange functionality can be provided by applying quaternary amines. Weak cation functionality can be produced by the use of carboxylic groups and weak anion functionality can be obtained by the use of primary amines.

Sphericity of the particles, rather than irregular, granular shapes, may be preferred, for providing minimum resistance to flow through a packed bed of the particles and minimum back-pressure. Such regularly shaped particles are less likely to undergo densification during use.

Particle size and size distribution are also important properties of the particles of the invention. In general, particles larger than about 20 μm facilitate lower back-pressure in packed columns. Moreover, the chromatographic peak width and peak shape obtained with larger particles are usually wider than the peak width and shape obtained with particles in the range of 3-15 μm. Narrow peak shapes are frequently desired for many types of separations.

The available surface area of polyfluorinated particles produced by the method of the invention is ordinarily preferred to be at least about 200 m^2/g, in order to obtain higher loading of antigens on the particulate medium. Nevertheless, media having much lower surface areas can readily be made according to the invention by changing the amount of porogen used in the polymerization system and decreasing the size of the particles. A large pore volume, of at least 0.5 mL/g, is needed in order to obtain a high surface area.

A wide range of pore sizes can be available for different chromatographic procedures. Large pores are needed for the efficient capture of larger molecules, such as proteins, while small pores are needed for the efficient capture of small molecules. In general, the range of pore sizes may extend from below 60Å to as high as 1,000Å, 300-800Å being preferred. This range of sizes is quite readily available using the invention method of adjusting the relative amount and type of porogen within the formed polymer particles.

Because of the wide range of pH values at which chromatography media are used and because of the very high pH ranges that are encountered frequently to clean and regenerate them, it is necessary that they be chemically inert throughout the entire range of such pH exposures. In particular, chromatographic media must be able to withstand the high pH (12 or higher) encountered by the use of NaOH for cleaning the media particles, typically 0.1-1 normal.

The adsorbent particles used in the invention are quite versatile and may be used as the stationary phase for carrying out a wide variety of chromatographic separations. Examples of the chromatographic separations contemplated include reverse phase separations, affinity separations, expanded bed separations, ion-exchange chromatography, gel filtration, chromatographic component separation, solid phase extraction, filtration and other recognised technical methods of distinguishing, measuring or collecting components of a chemical, biological or physical mixture. The particles may be used as support for grafting different types of ligands. The particles are particularly suited for use where the sample to be chromatographed is DNA, RNA or polypeptides. The desired product can be selectively eluted by use of the appropriate buffer, at the appropriate pH, as is known to those of ordinary skill in the art.

The surface of the uncoated particles used in Example 7 is hydrophobic, and may have slight polarity, which combination of properties is ideal for reverse phase chromatographic separations. A substantial advantage of the polyfluorinated particles of the invention is that they do not have the limitations of silica, in reverse phase chromatography at least.

Advantageously, the polyfluorinated compounds of the invention may be used in medical devices with or without ligands on their surfaces to do separations that are not classified as chromatographic. For example, components of blood can be separated using a medical device in which the blood is pumped through a cartridge extra-corporeally and returned to the body. A component such as a toxin can be removed and not returned to the body.

Due to the stability of the polyfluorinated particles of the invention, sterilization can be done by gamma irradiation without destroying the particles. This property makes the particles particularly well suited for uses in medical devices that must be sanitized.

Example 1

A porous copolymer of divinylbenzene and pentafluorostyrene was prepared according to the procedure described in Example 1 of WO 00/77081, mixing together 13.0 g of divinylbenzene and 87.0 g of pentafluorostyrene. This procedure was also very effective in making spherical porous particles of pentafluorostyrene.

A plasmid (Amp-resistant) transformed host (DH5-alpha) was grown to high density in an enriched medium and the bacterial pellet was subjected to an alkaline lysis procedure. The lysate was filtered and then precipitated with 0.7 volumes of ice-cold

isopropyl alcohol (IPA) by centrifugation at 8000 x g for 45 minutes. The liquid from the centrifugation was used as the sample to be chromatographed.

A Vantage-L series column (4.4 cm id) was packed with an ethanolic slurry containing approximately 90 ml of the particles described above (50 µm, surface area of 300/m/g, non-PVA coated). The column was packed at about 20 ml/mm (approximately 80 cm/h linear flow rate) and operated at 16 ml/mm. Column effluent was monitored at 260 nm and the absorbance was detected on a chart recorder. The column was equilibrated with EQB (0.1 M potassium phosphate, pH 7, 2 mM tetrabutylammonium phosphate (TBAP) and 1% ethanol) and the above described sample to be chromatographed (30 mg worth) was not loaded until the pH of the effluent was less than 9. The wash buffer WBJ was 93% sodium chloride/TRIS/EDTA, pH 8, and 7% ethanol. The elution buffers were the following: EL1 (elution buffer 1) was 0.1 M potassium phosphate, 2 mM TBAP, 10% ethanol; EL2 (elution buffer 2) was 0.1 M potassium phosphate, 2 mM TBAP, 12.5% ethanol; and EL3 (elution buffer 3) was 0.1 M potassium phosphate, 2 mM TBAP, 10% ethanol.

Eluant analysis:

Sample 1 was collected during the load and re-equilibration step. No DNA was present on particles in the packed column.

Sample 2 was collected while WB1 was passing through the column and contained the bulk of the RNA and a small amount of nicked open circular DNA.

Samples 3 and 4 were collected during WB1. Sample 3 contained a small amount of supercoiled DNA, more nicked/open circular DNA and the last of the RNA. Sample 4 contained a small quantity of DNA.

DNA loss may be reduced by cutting back on the ethanolic content of WB1 or increasing the TBAP concentration, the latter of which is preferred since this may still allow species selectivity by ethanol concentration at samples 4, 5 and 7.

Sample 5 (EL1) contained supercoiled DNA and trace amounts of non-supercoiled.

Sample 6 (EL2) contained the bulk of the DNA of which more than 90% was supercoiled.

Sample 7 (EL3) contained the residual DNA of which at least 25% was non-supercoiled.

95% of the DNA in the plasmid was recovered.

The amount of endotoxin in Sample 6 was measured by LAL test (BioWhittaker) and found to be 58 EU/mg of DNA. The starting endotoxin level in the plasmid was 8000 EU/mg.

This example shows that the use of ion-pairing chromatography with fluorine-containing particles can isolate super-coiled DNA and concurrently lower the endotoxin level in the DNA.

The RNA in Sample 2 can be further purified. This example also shows that this method and materials can also be used to isolate RNA from a plasmid.

Example 2

Six litres of LB medium spiked with Ampicillin (50 μg ml^{-1}) was inoculated with 2.5 ml of a seed culture of pRc/CMV-HBs(s) (ca. 5500 bp) bearing host *(E. coli* DH5α) and grown for 13 hours. The cell mass was harvested by centrifugation. The material was resuspended in 500 ml of 50 mM Tris.HCl/10 mM EDTA (pH 8) containing 100 μg ml^{-1} RNaseA. An equal volume of lysis buffer (1% SDS/0.2 N NaOH) was added and the contents were mixed by inversion and allowed to stand for five minutes. Then, 125 ml of ice-cold neutralization buffer (3 M potassium acetate, pH 5.5) was added to the mixture and a white precipitate formed.

The mixture was immediately centrifuged for 20 minutes at 9,000 x g (4°C). Ice-cold 2-propanol was applied to the lysate. The mixture was spun for 15 minutes at 5,000 x g (4°C). The pellet was allowed to air dry and was then suspended in buffer A (a water solution of triethylamine acetate at pH 7.0).

Ion-pairing chromatography was performed as follows: 24 g of a granular tetrafluorinated ethylene polymer was placed in a column of 38 mm (1.5 inches) I.D. and 152 mm (6.0 inches) length with a capacity of 300 cc. The bed height was 51 mm (2 inches). The polymer was equilibrated with buffer A. Then, 40 ml of the re-suspended, lysed plasmid in buffer A was allowed to flow through the column. Then, two washes using 40 ml of buffer B were done. Buffer B contained a mixture of Tris, EDTA and sodium chloride. The column was then treated with 50 ml of buffer C. Buffer C contained sodium acetate and isopropyl alcohol.

The RNA was eluted in the first wash buffer B. No DNA was detectable in the eluent when buffer B was used. The eluent from buffer C contained the DNA in supercoiled, relaxed and linear states.

The elution fraction using buffer C was split into centrifuge tubes) and precipitated using centrifugation at 8,000 x g (4°C) for 20 minutes using ice-cold, absolute ethanol. The pellets were pooled by combining them using 70% ethanol, spun

briefly and then allowed to air dry. 3 ml of TE (10 mM Tris/1 mM EDTA, pH 8) were added to the pellets and they were gently agitated at room temperature to re-suspend the DNA. The DNA was then tested for composition and purity.

The quantity of the DNA was 8.4 and 9.1 mg from duplicate experiments. The purity of the DNA, as measured by ultra-violet analysis using the ratio of 260/280 nm wavelengths was 1.54 and 1.81 respectively, indicating a high level of purity. The amount of RNA in the DNA was undetectable, using a BCA assay from Pierce Chemical Corporation. The amount of endotoxin in the DNA as measured by a BioWhittaker QLC test was 58.8 EU/mg and 64.4 EU/mg in duplicate tests. The starting amount of endotoxin in the plasmid was >8000 EU/mg.

Example 3

A plasmid was made using the above fermentation procedure and purified using ion-exchange and size exclusion chromatography, to give DNA with a 260/250 mn ratio of 1.81 indicating that it was very pure and contained virtually no RNA. In an attempt to quantify the adsorption/desorption of DNA onto its surface, 16 g of a granular polytetrafluoroethylene homopolymer of 30 µm particle size was placed into a chromatography column. 4.0 mg of DNA described above was loaded onto the column using the procedure described in Example 2. Using the methods described in Example 2, it was found that 4.0 mg was adsorbed onto the column and 3.90 mg was eluted from the column using buffer C. This represents 97.5% of the DNA. This shows the affinity of the totally fluorinated polymer for DNA and the ability to desorb all of the DNA using ion-pairing chromatography. The flow time was 1.5 hours.

Example 4

Example 3 was repeated, except that the polymer used in the column was a granular copolymer of tetrafluoroethylene and perfluoropropyl vinyl ether. The particle size was 30 µm. 82% of the DNA was adsorbed onto the column, indicating that polar groups may interfere with adsorption and desorption. The flow time was 1.5 hours.

Example 5

Example 3 was repeated, except that a granular copolymer of particle dimension 30 µm of tetrafluoroethylene and hexafluoroethylene (12/1) was used in the column. in this case 58% of the polymer was adsorbed and eluted, with a flow time of I.5 hours. This shows that there may be a negative effect on yield of dilution of the fluorine content of the polymer.

Example 6

Example 3 was repeated, except that a granular copolymer of particle dimension of 30 µm of tetrafluoroethylene and ethylene (1/1) was used in the column. In this case 39% of the DNA was adsorbed and eluted with buffer C. The flow time was 1.5 hours.

Example 7

Example 3 was repeated, except that a copolymer of ethylene glycol dimethacrylate and pentafluorostyrene (85/15 w/w) of particle size 30 µm and pore diameter of 700 Å was used. 95% of the DNA was adsorbed and desorbed. The flow time was 3.25 minutes.

Example 8

Example 3 was repeated, except that spherical copolymer particles of pentafluorostyrene and ethylene glycol dimethacrylate (76/24) of particle size of 60 µm and pore size of 700 Å was used. In this case only 65% of the DNA was adsorbed and desorbed. The flow time was 1.2 minutes.

Example 9

Example 3 was repeated, except that a copolymer of pentafluorostyrene and ethylene glycol dimethacrylate and hydroxymethyl methacrylate (40/28/28) was used. The spherical particle size was 60 µm and the pore size was 600 Å. 15% of the DNA was adsorbed and desorbed. The flow time was 1.5 hours.

This Example indicates that polar groups and dilution of the fluorine content of the polymer have affected the purity of the DNA. This Example also shows that copolymers can be made which have the functionality for reacting with attachment ligands such as epoxide.

Example 10

Example 3 was repeated, except that a 50/50 by weight of a mixture of the polymers in Examples 4 and 8 was used. The yield was 88.0 % and the flow time was 6.93 minutes. This shows that mixtures can be used to optimize residence time and yield.

Example 11

Example 3 was repeated, except that a copolymer of styrene and divinylbenzene of particle size 35 µm and 1000 Å pore size (Amberchrome purchased from TosoHaas) was used. All of the DNA was absorbed onto the polymer but none could be eluted using the method above. This shows the necessity of having fluorine atoms on the polymer, in order to be able to elute DNA using ion-pairing chromatography.

CLAIMS

1. A method for the separation of a nucleic acid in a liquid sample, comprising passing the sample through a bed of adsorbent particles of a fluoropolymer, and elution of a desired fraction, wherein the method comprises the use of an ion-pairing component.

2. The method of claim 1, wherein the sample comprises the lysis products of a plasmid.

3. The method of claim 1 or claim 2, wherein the sample comprises endotoxins.

4. The method of any preceding claim, for separating RNA.

5. The method of any preceding claim, for separating DNA.

6. The method of any preceding claim, conducted in the absence of any polar components.

7. The method of any preceding claim, wherein the particles comprise granular and/or spherical particles.

8. The method of any preceding claim, wherein the particles are not cross-linked.

9. The method of any of claims 1 to 7, wherein the particles are cross-linked with divinylbenzene.

10. Adsorbent particles of a fluoropolymer having affinity ligands bound thereto, obtainable by copolymerisation of monomers including a comonomer having a functional group, and attaching the affinity ligands to the particles *via* the functional group.

INTERNATIONAL SEARCH REPORT

Inte onal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 B01D15/08 B01J20/26 C07H1/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N B01D B01J C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; November 1998 (1998-11) KAPUSTIN D V ET AL: "Composite fluoropolymer-containing sorbents for isolation and purification of biopolymers." Database accession no. PREV199900110788 XP002178777 abstract & BIOORGANICHESKAYA KHIMIYA, vol. 24, no. 11, November 1998 (1998-11), pages 868-876, ISSN: 0132-3423 --- -/--	1,2,4,5, 7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

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